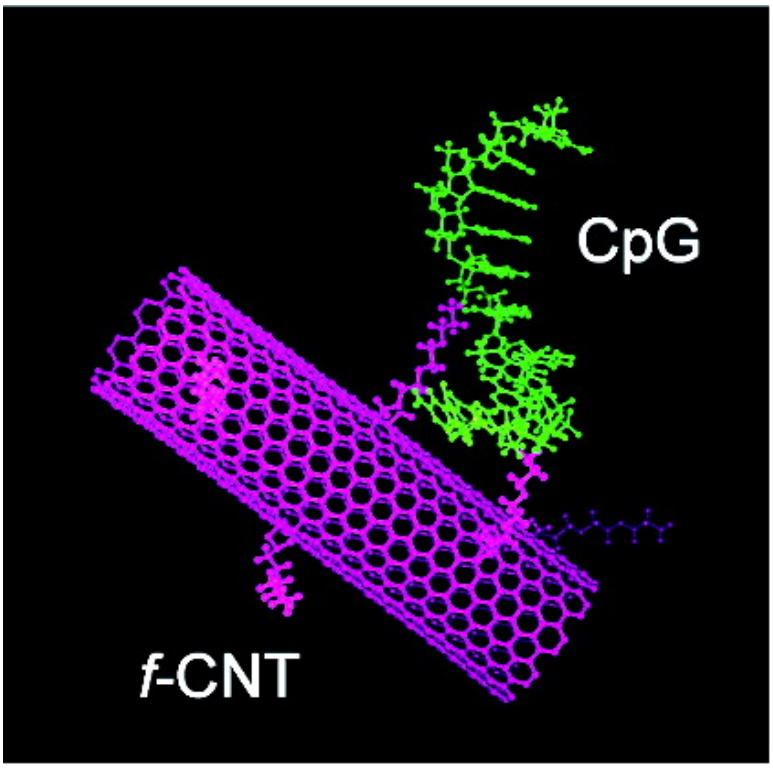
Communication

# Cationic Carbon Nanotubes Bind to CpG Oligodeoxynucleotides and Enhance Their Immunostimulatory Properties

Alberto Bianco, Johan Hoebeke, Sylvie Godefroy, Olivier Chaloin, Davide Pantarotto, Jean-Paul Briand, Sylviane Muller, Maurizio Prato, and Charalambos D. Partidos

*J. Am. Chem. Soc.*, **2005**, 127 (1), 58-59• DOI: 10.1021/ja044293y • Publication Date (Web): 16 December 2004






## More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 9 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article

J|A|C|S
COMMUNICATIONS

Published on Web 12/16/2004

# Cationic Carbon Nanotubes Bind to CpG Oligodeoxynucleotides and Enhance Their Immunostimulatory Properties

Alberto Bianco,*,† Johan Hoebeke,† Sylvie Godefroy,† Olivier Chaloin,† Davide Pantarotto,†,‡ Jean-Paul Briand,† Sylviane Muller,† Maurizio Prato,*,‡ and Charalambos D. Partidos*,†

*Institut de Biologie Moléculaire et Cellulaire, UPR9021 CNRS, Immunologie et Chimie Thérapeutiques, 67084 Strasbourg, France, and Dipartimento di Scienze Farmaceutiche, Università di Trieste, 34127 Trieste, Italy*

Received September 20, 2004; E-mail: A.Bianco@ibmc.u-strasbg.fr; H.Partidos@ibmc.u-strasbg.fr

Several studies have documented that bacterial DNA constitutes a "danger signal" for the immune system and has immunostimulatory properties.[1] This has been attributed to the presence of unmethylated CpG motifs, which are far more common in bacterial DNA than in vertebrate DNA.[1] The activation of the immune system by CpG motifs is achieved through the Toll-like receptor (TLR) 9, which is localized intracellularly.[1−3] They directly activate antigen presenting cells to release Th1 cytokines and express costimulatory molecules.[1]

Synthetic oligodeoxynucleotides containing CpG motifs (ODN CpGs) confer nonspecific protection against various intracellular pathogens and enhance antigen-specific immune responses. Therefore, they have long been considered as candidate adjuvants for vaccines or immunomodulators for therapeutic applications against tumors, allergies or to combat bioterrorist threats.[4−7] However, their biological activities are often short-lived, and therefore, several administrations of a high dose are normally required. This is because the intracellular delivery of ODN CpGs faces the challenge of low uptake by the cells because of the negative charge of cell membranes.[8]

Cationic phospholipids and cationic polymers are two major delivery vectors for ODN CpGs that are currently under investigation.[9,10] Their charge permits the interaction with the negatively charged ODN CpG. Cationic-functionalized carbon nanotubes (*f*-CNT) could represent a valuable alternative vector to use because of their capacity to enter into the cells.[11−16]

In a recent report, we demonstrated that ammonium-functionalized single-walled carbon nanotubes could successfully condense DNA and achieve significant transfection in vitro.[17] In this study, we selected two types of cationic *f*-CNT (Table 1) to evaluate their interaction with one specific ODN immunostimulatory CpG motif (CpG 1668; see Supporting Information for its sequence) using surface plasmon resonance technology (SPR).

SPR measurements allow the analysis of interaction between two macromolecules in real time.[18] *f*-CNT **1** and **2** were covalently linked to the carboxylic groups of the dextrane matrix covering the sensor chip, previously activated with 1-hydroxysuccinimide and *N*-ethyl-*N*′-dimethylaminopropyl carbodiimide. The increase in mass due to the interaction of *f*-CNT **1** and **2** with the ODN CpG 1668 present in the fluid phase was then measured (Supporting Information). Kinetic analysis of the sensorgrams (Figure S1) revealed that the association rate constant was higher for the *f*-CNT **2** as compared to that of the *f*-CNT **1** (Table 1). Similarly, the *f*-CNT **1** showed a slightly faster dissociation process as compared to that of the *f*-CNT **2**. This could be due to the avidity factor of the bivalent ligand **2**. There were no major differences in the binding affinity of ODN CpG 1668 to either *f*-CNT (Table 1).

† Institut de Biologie Moléculaire et Cellulaire.
‡ Università di Trieste.

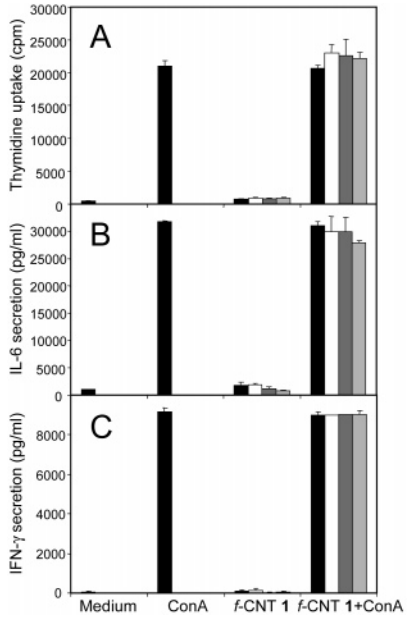



***Figure 1.*** (A) Thymidine uptake, (B) IL-6, and (C) IFN-$\gamma$ secretion by ConA-activated or nonactivated mouse splenocytes cultured with various concentrations [5 $\mu$g (black), 0.5 $\mu$g (white), 0.05 $\mu$g (dark gray), and 0.005 $\mu$g (light gray)] of *f*-CNT **1**.

***Table 1.*** Kinetics and Affinity Constants for the Interaction of *f*-CNT with ODN CpG

| | *f*-CNT | | |
|---|---|---|---|
| | | **1** | **2** |
| $k_{on}$ [$M^{-1}\cdot s^{-1}$] | | $(5.19 \pm 1.55) \times 10^5$ | $(1.23 \pm 0.14) \times 10^6$ |
| $k_{off}$ [$s^{-1}$] | | $(2.99 \pm 1.46) \times 10^{-3}$ | $(4.85 \pm 0.35) \times 10^{-3}$ |
| $K_d$ [M] | | $(5.88 \pm 2.30) \times 10^{-9}$ | $(3.99 \pm 0.66) \times 10^{-9}$ |

No toxic effects of *f*-CNT on mitogen activated (ConA, Conconavalin A) and nonactivated mouse splenocytes were observed. Splenocytes from naïve mice were recovered, cultured in the presence of increasing concentrations of *f*-CNT alone or together with a constant amount of ConA, and finally pulsed with $^3$[H]-thymidine (Supporting Information). Proliferation was measured by counting tritium radioactivity in each culture.

Figure 1A shows a representative experiment where different concentrations of *f*-CNT **1** had no significant effect on the $^3$[H]-thymidine uptake by activated or nonactivated splenic lymphocytes. Moreover, they did not affect the concentration of secreted IL-6 (Interleukin-6) (Figure 1B) or IFN-$\gamma$ (Figure 1C) measured in


10.1021/ja044293y CCC: $30.25 

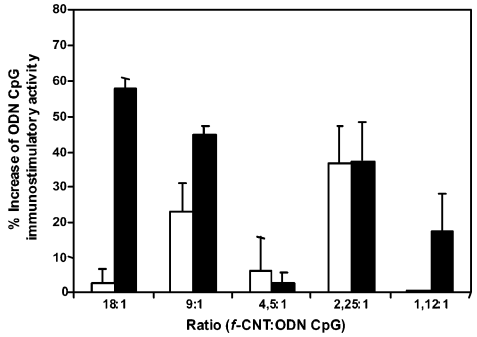



***Figure 2.*** Percentage increase of the immunostimulatory activity of ODN CpG 1668 when complexed with the *f*-CNT **1** (open bars) or *f*-CNT **2** (solid bars) formulations. The background level of $^3$[H]-thymidine uptake in cultures with medium alone was 359 cpm, whereas in the uptake in the presence of ODN CpG 1668 it was 5571 cpm.

culture supernatants of mitogen-stimulated splenocytes using ELISA kit (Supporting Information). No IL-6 or IFN-$\gamma$ secretion was detected in supernatants of nonactivated mouse splenocytes in the presence of various concentrations of *f*-CNT **1**.

To test the ability of *f*-CNT to increase the immunostimulatory properties of ODN CpG, a constant amount of ODN CpG 1668, which in titration experiments on mouse splenocytes was shown to be the minimum immunostimulatory dose (0.05 $\mu$g/culture), was complexed with different concentrations of *f*-CNT **1** or **2** (based on the available surface charge), giving an excess of 18:1, 9:1, 4.5:1, 2.25:1, and 1.12:1 of *f*-CNT over the ODN CpG 1668. Following incubation of the complexes with mouse splenocytes for 3 days, a modest increase of the immunostimulatory activity was observed that reached 23 and 37% when using the *f*-CNT **1** formulation at 9:1 and 2.25:1 ratio, respectively (Figure 2). However, the immunostimulatory activity of the ODN CpG 1668 had a more pronounced increase when it was complexed with the *f*-CNT **2**, reaching up to 58 and 45% at 18:1 and 9:1 ratio, respectively (Figure 2). Complexes of both *f*-CNT **1** and **2** with the ODN containing the nonimmunostimulatory CpG motif 1982 (Supporting Information for its sequence) had no activity on lymphocytes (data not shown).

Since ODN CpG 1668 is known to stimulate the production of proinflammatory cytokines,[19] we measured levels of IL-6 in supernatants of splenocyte cultures stimulated with ODN CpG 1668 alone or complexed with different ratios of *f*-CNT. Interestingly, IL-6 secretion was decreased in cultures stimulated with *f*-CNT **1**/ODN CpG 1668 complexes by 94% at 18:1, 86% at 9:1, 56% at 4.5:1, 50% at 2.25:1, and 2.7% at 1.12:1 *f*-CNT/ODN CpG ratio and to a lesser extent with *f*-CNT **2**/ODN CpG 1668 complexes (21% at 18:1, 20% at 9:1, 6.44% at 4.5:1, 5.14% at 2.25:1, and 9% at 1.12:1 *f*-CNT/ODN CpG ratio) as compared to those levels produced by the ODN CpG 1668 alone.

The results of this study demonstrate the potential of *f*-CNT to improve the immunostimulatory properties of ODN CpGs in vitro. The observed immunopotentiating effect is likely to be due to the high loading capacity of *f*-CNT **2** ($R_{max}$ for *f*-CNT **1** and **2** was 21.3 and 125 RU, respectively; see Supporting Information) and cell-penetrating ability. The ratio between both substances was found to be critical for improving immunostimulation. It could be argued that the excess of *f*-CNT **2** neutralizes the negative charge of ODN CpG. As a consequence, the repulsion by the negatively charged cell membrane is presumably reduced, and therefore, the cellular uptake of ODN CpG is facilitated. The observed increase of the immunostimulatory capacity of ODN CpG 1668 when complexed with *f*-CNT **2** was not accompanied by a parallel enhancement of IL-6 secretion, a proinflammatory cytokine that can cause harm to the host. This finding is consistent with observations demonstrating that complexes of ODN CpGs with cationic peptides elicit reduced production of proinflammatory cytokines as compared to those they normally stimulate alone.[20] In our studies, we also demonstrated that *f*-CNT do not exert any mitogenic nor any toxic effect on activated or nonactivated lymphocytes. This finding is in contrast to previously published work demonstrating the toxicity of non *f*-CNT, which are an insoluble material.[21]

In conclusion, these findings suggest that *f*-CNT could be advantageous for the effective delivery of ODN CpGs into target cells. However, further work is required to evaluate the immunopotentiating capacity of these complexes in vivo using animal models of disease or vaccination protocols to test their therapeutic or adjuvant properties, respectively.

**Acknowledgment.** This work was financially supported by the CNRS, the University of Trieste, and MIUR (PRIN 2004, prot. 2004035502). We wish to thank Felix Hoffman and Nelmar Zorianny Garcia for excellent technical assistance. Ken A. Smith from Carbon Nanotechnologies, Inc. is also greatly acknowledged for the generous gift of carbon nanotubes.

**Supporting Information Available:** Details on the functionalization of CNT, surface plasmon resonance, assay for lymphocyte stimulation, and cytokine measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Krieg, A. M. *Annu. Rev. Immunol.* **2002**, *20*, 709−760.
(2) Cornelie, S.; Hoebeke, J.; Schacht, A. M.; Bertin, B.; Vicogne, J.; Capron, M.; Riveau, G. *J. Biol. Chem.* **2004**, *279*, 15124−15129.
(3) Ahmad-Nejad, P.; Hacker, H.; Rutz, M.; Bauer, S.; Vabulas, R. M.; Wagner, H. *Eur. J. Immunol.* **2002**, *32*, 1958−1968.
(4) McCluskie, M. J.; Weeratna, R. D.; Davis, H. *Vaccine* **2001**, *19*, 2657−2660.
(5) Davila, E.; Celis, E. *J. Immunol.* **2000**, *165*, 539−547.
(6) Sur, S.; Wild, J. S.; Choudhury, B. K.; Sur, N.; Alam, R.; Klinman, D. M. *J. Immunol.* **1999**, *162*, 6284−6293.
(7) Klinman, D. M.; Verthelyi, D.; Takeshita, F.; Ishii, K. J. *Immunity* **1999**, *11*, 123−129.
(8) Mutwiri, G. K.; Nichani, A. K.; Babiuk, S.; Babiuk, L. A. *J. Controlled Release* **2004**, *97*, 1−17.
(9) Singh, M.; Ott, G.; Kazzaz, J.; Ugozzoli, M.; Briones, M.; Donnelly, J.; O'Hagan, D. T. *Pharm. Res.* **2001**, *18*, 1476−1479.
(10) Babiuk, S.; Baca-Estrada, M. E.; Middleton, D. M.; Hecker, R.; Babiuk, L. A.; Foldvari, M. *Curr. Drug Delivery* **2004**, *1*, 9−15.
(11) (a) Pantarotto, D.; Briand, J.-P.; Prato, M.; Bianco, A. *Chem. Commun.* **2004**, 16−17. (b) Shi Kam, N. W.; Jessop, T. C.; Wender, P. A.; Dai, H. *J. Am. Chem. Soc.* **2004**, *126*, 6850−6851.
(12) Georgakilas, V.; Tagmatarchis, N.; Pantarotto, D.; Bianco, A.; Briand J.-P.; Prato, M. *Chem. Commun.* **2002**, 3050−3051.
(13) Tasis, D.; Tagmatarchis, N.; Georgakilas, V.; Prato, M. *Chem.−Eur. J.* **2003**, *9*, 4000−4008.
(14) Pantarotto, D.; Partidos, C. D.; Graff, R.; Hoebeke, J.; Briand, J.-P.; Prato, M.; Bianco, A. *J. Am. Chem. Soc.* **2003**, *125*, 6160−6164.
(15) Pantarotto, D.; Partidos, C. D.; Hoebeke, J.; Brown, F.; Kramer, E.; Briand, J.-P.; Muller, S.; Prato, M.; Bianco, A. *Chem. Biol.* **2003**, *10*, 961−966.
(16) (a) Bianco, A.; Prato, M. *Adv. Mater.* **2003**, *15*, 1765−1768. (b) Lin, Y.; Taylor, S.; Li, H.; Fernando, K.; Qu, L.; Wang, W.; Gu, L.; Zhou, B.; Sun, Y.-P. *J. Mater. Sci.* **2004**, *14*, 527−541.
(17) Pantarotto, D.; Singh, R.; McCarthy, D.; Erhardt, M.; Briand, J.-P.; Prato, M.; Kostarelos, K.; Bianco, A. *Angew. Chem., Int. Ed.* **2004**, *43*, 5242−5246; *Angew. Chem.* **2004**, *116*, 5354−5358.
(18) Baird, C. L.; Myszka, D. G. *J. Mol. Recognit.* **2001**, *14*, 261−268.
(19) Lipford, G. B.; Sparwasser, T.; Bauer, M.; Zimmermann, S.; Koch, E. S.; Heeg, K.; Wagner, H. *Eur. J. Immunol.* **1997**, *27*, 3420−3426.
(20) Lingnau, K.; Egyed, A.; Schellack, C.; Mattner, F.; Buschle, M.; Schmidt, W. *Vaccine* **2002**, *20*, 3498−3508.
(21) Colvin, V. L. *Nat. Biotechnol.* **2003**, *21*, 1166−1170.

JA044293Y